COLORIMETRIC SCREENING OF MITRAGYNINE IN BIOLOGICAL AND NON-BIOLOGICAL SAMPLE MATRICES FOR DETECTION OF FORENSIC DRUGS

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by

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Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science (Forensic Science)

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CERTIFICATE

This is to certify that the dissertation entitled **"Colorimetric Screening of Mitragynine in Biological and Non-Biological Samples Matrices for Detection of Forensic Drugs"** is the bona fide record of research work done by Miss Nur Shahida Binti Mohamad Zamri under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the Master of Science (Forensic Science).

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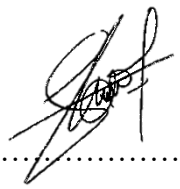
DECLARATION

I hereby declare that this dissertation is the result of my own investigation, excepts where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other master at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.



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LIST OF ABBREVIATIONS

BRM	Botanical reference material
EPS	Emerging Psychoactive Substance
GC/MS	Gas chromatography/mass spectrometry
GC	Gas chromatography
GC-FID	Gas chromatography-Flame ionization detector
HCL	Hydrochloric acid
HUSM	Hospital Universiti Sains Malaysia
HPTLC	High performance thin layer chromatography
HPLC	High performance liquid chromatography
INFORMM	Institute for Research in Molecular Medicine
iOS	iPhone Operating system
JEPeM	Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia
LC-MS	Liquid chromatography- mass spectrometry
LC-ESI-MS	Liquid chromatography-electrospray ionization mass spectrometry
LSD	Lysergic acid diethylamine
MG	Mitragynine
NaOH	Sodium hydroxide
NDARC	National Drug and Alcohol research centre
NPS	New Psychoactive Substances
PTPE	Polytetrafluoroethylene
p-DMAB	para-dimethylaminobenzaldehyde
TLC	Thin layer chromatography
UNODC	United Nation on Drugs and Crime
UK	United Kingdom
US	United State
UPLC-MS/MS	Performance liquid chromatography-tandem mass spectrometry
VU	Van Urk

LIST OB SYMBOLS

cm	Centimetre
^{o}C	Degree Celsius
g	Gram
kg	Kilogram
mL	Millimetre
mg/mL	Milligram per millimetre
µL	Microliter
nm	Nanometre

PENYARINGAN REAKSI WARNA UNTUK MITRAGINA DALAM SAMPLE MATRIKS BIOLOGI DAN BUKAN BIOLOGI UNTUK PENGESANAN DADAH FORENSIK.

ABSTRAK

Bahan psikoaktif baru boleh didefinasikan sebagai bahan salah guna atau lebih dikenali sebagai 'legal high' atau 'garam mandian' menurut United Nation on Drugs and Crime (UNODC). Tersenarai dalam bahan psikoaktif baru, mitragina merupakan sebatian yang mempunyai ciri-ciri opioid yang terkandung dalam pokok *Mitragyna speciose* atau lebih dikenali sebagai pokok ketum. Ketum adalah pokok yang tergolong dalam keluarga pokok kopi yang asli daripada Asia Tenggara termasuk Malaysia, Thailand, Indonesia dan Burma. Secara amnya, kajian ini tertumpu kepada menyiasat tindak balas warna mitragina terhadap bahan uji Van Urk dalam sampel biologi dan bukan biologi untuk pemerhatian visual dan analisis spektrofotometer. Ujian warna dengan bahan uji Van Urk dan kolorimetri (aplikasi telefon bimbit dan spektrofotometer pembaca mikroplat) telah dijalankan untuk mengesan mitragina di dalam pelbagai sampel (biologi dan bukan biologi). Pengesanan mitragina daripada sampel rebusan, konkosi dan sample serbuk dengan bahan uji Van Urk menghasilkan tindak balas yang pantas dan berjaya menunjukkan perubahan warna kepada merah jambu. Namun begitu, sampel biologi seperti air kencing menunjukkan keputusan yang tidak muktamad di mana tiada perubahan warna merah jambu yang ketara apabila pengesanan terus menggunakan bahan uji Van Urk dilakukan. Penyarian perlu dilakukan terhadap sampel biologi terutamanya sample air kencing untuk memperoleh keputusan yang lebih baik. Untuk meringkaskan, analisis tindak balas warna untuk pengukuran gandaan sampel menggunakan telefon pintar sebagai pengesan adalah tidak ideal. Nilai RGB yang diukur adalah tidak konsisten walaupun sistem

penangkapan imej yang standard dibina untuk menangkap gambar semua sampel. Tambahan pula, analisis spektrofotometer menggunakan pembaca mikroplat juga tidak menunjukkan keputusan yang memuaskan.

COLORIMETRIC SCREENING OF MITRAGYNINE IN BIOLOGICAL AND NON-BIOLOGICAL SAMPLES MATRICES FOR DETECTION OF FORENSIC DRUGS

ABSTRACT

New psychoactive substances (NPS) can be defined as the substance of abuse or been known as 'legal high' or 'bath salts' according to the United Nation on Drugs and Crime (UNODC). Listed as NPS, mitragynine is a compound that has the opioid type properties that are present in *Mitragyna speciosa* plant or also known locally as ketum plant. Ketum' is a plant in the coffee family plant native to Southeast Asia including Malaysia, Thailand, Indonesia and Burma. Generally, this study focuses on investigating mitragynine colour reaction towards Van Urk reagent in biological and non-biological samples by visual observation and spectrophotometric analysis. Colour test with Van Urk reagent and colorimetric analyses (mobile application and microplate reader spectrophotometer) were conducted for detection of mitragynine in various sample matrices (biological and non-biological). The detection of mitragynine from decoction, concoction and powder (non-biological) samples with Van Urk reagent produced a fast and successful reaction of colour change to pink. However, as for biological sample such as urine, direct detection using Van Urk reagent shown inconclusive result which there was no apparent of pink colour change. Extraction needs to be performed on biological samples, precisely urine sample in order to produce a better result. To summarise, the colorimetric analysis of multiple-sample measurement using a smartphone a detector was not ideal. RGB values that measured was not consistent even though the standardised image capture system was built to

capture the image of all the samples. Furthermore, spectrometric analysis using microplate reader spectrophotometer also did not convey satisfactory result.

CHAPTER 1

INTRODUCTION

1.1 Background of study

New psychoactive substances (NPS) can be defined as the substance of abuse or been known as 'legal high' or 'bath salts' according to the United Nation on Drugs and Crime (UNODC). NPS have widely spread and become a global phenomenon for more than 100 countries and territories reported one or more NPS. NPS is categorized in a few groups such as aminoindanes, phencycylidine-type substances, phenethylamines, piperazines, plant-based substances, synthetic cannabinoids, synthetic cathinones, tryptamines and other substances (United Nation on Drugs and Crimes (UNODC), 2020).

Listed as NPS, mitragynine is a substance that is present in *Mitragyna speciosa* plant or also known locally as ketum plant. Ketum is a plant in the coffee family plant native to Southeast Asia including Malaysia, Thailand, Indonesia and Burma. In Ketum leaves, there are compounds that have the opioid type properties which are mitragynine and 7-hydroxymitragynine. These compounds have similar properties with morphine and heroin.

In Malaysia, ketum is regulated under Poison act 1952 in Section 3(3) which states that importing, exporting, manufacturing, supplying, selling or in possession of ketum illegally can be fined up to RM10, 000 or face four years of imprisonment or both. Consuming or planting ketum is not a crime; however, ketum abuse among teenagers and youngsters is increasing day by day for the past few decades. The trend shifted from traditional medicine to abuse among youngsters. Youngsters in the age between 12 to 18 years old who is still in the school had been introduced to ketum.

Traditionally, ketum was prepared by brewing the leaves in the large pot of water without adding other substances, but nowadays, ketum users had altered and added other substance such as coke, cough syrup for an extra kick (Tanguay, 2011).

In the last decades, Malaysia has seen a sharp rise in the addiction and seizure of herbal new psychoactive substances (NPS), especially from *Mitragyna speciosa* (ketum) origin. The standard forensic chemical testing protocol requires two steps identification procedures; preliminary test for any illegal substances prior to confirmatory analysis. Interestingly, the increase in ketum prevalence has revealed a lapse in current presumptive testing methodologies.

Colorimetric screening test to detect mitragynine (MG), a significant alkaloid responsible for psychoactive opioid effect in ketum are not prioritized possibly due to lack of understanding of colour reaction mechanism although it is highly desirable in accordance to the standard practice. The complexity of sample preparation to extract MG from various sample matrices and its reaction towards colour reagent need to be fully explored. This work will impact forensic science and regulatory agencies by fully understanding mechanisms of colour change in order to provide rapid and straightforward drug detection to deter ketum abuse among public society.

1.2 Problem Statement

Screening test for illegal substances prior to confirmatory analysis is a standard protocol for forensic drug detection, in order to identify any substance involved in forensic drug testing need to perform two types of analysis which are preliminary testing and followed by confirmatory analysis. Commonly, preliminary testing involves a physical examination, colour test and also thin layer chromatography analysis. Further analysis to identify the substance or compound needs to perform confirmatory analysis.

Mitragynine (MG) is one of the psychoactive substances that have become established on the drug market; however, it is not effectively discriminated or identified by current colour testing methods. The increase in ketum prevalence has revealed a lapse in current presumptive testing methodologies. In current practice, colorimetric screening test to detect mitragynine in non-biological (drinks or powdered samples) and biological matrices (urine samples of suspected users) are not conducted due to the standard existing reagent kit is not ideal as they only produce natural botanical (brown and green) colour post-reaction. The existing kit is only ideal for the detection of common drugs such as cannabis, heroin, LSD drugs but not mitragynine (ketum).

The use of Van Urk's (VU) reagent (1 percent p-dimethyl-aminobenzaldehyde in hydrochloric acid) has been suggested for colorimetric procedure for the detection of indoles, pyrroles and related nitrogen-containing compounds. Preliminary studies in our laboratory on VU colour test to MG has demonstrated a highly contrasting colour when reacted with MG. Detection of MG in non-biological and biological samples have produced a pink and a blue positive reaction, respectively. Thus the need to test

reagent specificity towards mitragynine compound in various matrices is highly warranted.

1.3 Objectives

In general, this study aims to investigate MG colour reaction towards Van Urk reagent in biological and non-biological samples by visual observation and spectrophotometric analysis.

1.3.1 Specific objectives

1. To study the reaction of Van Urk reagent to MG in aqueous samples.
2. To study the reaction of Van Urk reagent to MG in extracted samples.
3. To observe colour changes of various samples using spectrophotometric and mobile application for MG detection.

1.4 Research question

1. Can all samples (both biological and non-biological) show detection of MG using Van Urk reagent?
2. Does the result obtain from visual observation be comparable to the result produced by spectrophotometric method?
3. Do different concentrations of MG show different RGB values when analysed using mobile application?

1.5 Significant of study

Study may contribute towards on-site screening and detection of MG compound in both various sample matrices using colorimetric analysis (Van Urk colour test). The possibility of using portable spectrophotometer or mobile application is explored as if is convenient. Hence, the rapid and fast detection can apply to on-site detection of ketum.

CHAPTER 2

LITERATURE REVIEW

2.1 New psychoactive substances (NPS)

The term legal high or salt bath commonly term that popular for calling for new psychoactive substances or NPS. According to United Nations Office on Drugs and Crime, NPS is defined as substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat. Because of that, NPS may be prohibited in certain countries but not for other countries. NPS is regulated differently in different countries.

There is another term that related to the term NPS but have different in meaning which is the emerging psychoactive substances (EPS). The term new psychoactive substances cover all the new substances/ drugs invented in the market, however, some of them were present in the market for quite sometimes. Therefore, the National Drug and Alcohol research centre (NDARC) has introduced a new term i.e. emerging psychoactive substances (EPS) to capture psychoactive drugs that are relatively new to recreational drugs markets (NDARC, 2016).

NPS was categorized into different groups according to their properties and affect the human body. NPS was categorized mainly in four main groups; stimulants, cannabinoids, hallucinogens, and depressants. According to NDARC (2016), NPS was categorized in synthetic cannabinoids, phenethylamines, synthetic cathinones, tryptamines, Other groups including novel benzodiazepines, novel opioid, and plant-based NPS (plants with psychoactive properties) such as ketum, khat, and Salvia Divinorum.

Plant-based NPS are plants that contain psychoactive properties that may act as drugs substitute for far more dangerous and addictive drugs. Ketum was included in plant-based NPS due to the active compound (MG) which had opioid properties similar to morphine and heroin. NPS was regulated differently in different countries which explained certain country has banned the plants and others were not (Tanguay, 2011).

2.2 Mitragynine and 7-Hydroxymitragynine

For the past few decades, Ketum was used as traditional medicine for hard labour for suppress pain and stare off exhaustion as well as typical painkiller. Mitragynine is the active indole compounds and major constituent alkaloid in the *Mitragyna Speciosa* plant or commonly known as ketum plant. In ketum leaves consist of several alkaloids which are mitragynine 66.2%, paynantheine 8.6%, speciogynine 6.6%, 7- hydroxy-mitragynine 2.0% and speciociliatine 0.8% (Casey, et al., 2015). Mitragynine is the psychoactive substance that is dose dependent substance which similar to other psychoactive drugs. Both mitragynine and 7-hydroxymitragynine compounds are analgesic and opioid type of compound and will interact with opioid receptors in the brains and producing sedation, pleasure and decreased pain especially when users consume large amounts of plants. Mitragynine and 7-hydroxy-mitragynine would give a similar effect with compounds such as morphine and heroin.

Mitragynine and 7-hydroxy-mitragynine had similar chemical structures but the difference between mitragynine and 7-hydroxymitragynine is the presence of the hydroxyl group for 7-hydroxymitragynine. The chemical structure of both compounds shown in Figure 2.1. The studies from Ponglux, et al. (1994), they reinvestigate the alkaloid constituent in ketum plants in Thailand and obtained the new alkaloid in

ketum plants which was 7-hydroxymitragynine. From that research, they found out that the 7- hydroxymitragynine compound is the derivate of 7-hyrdroxyindolenine of mitragynine.

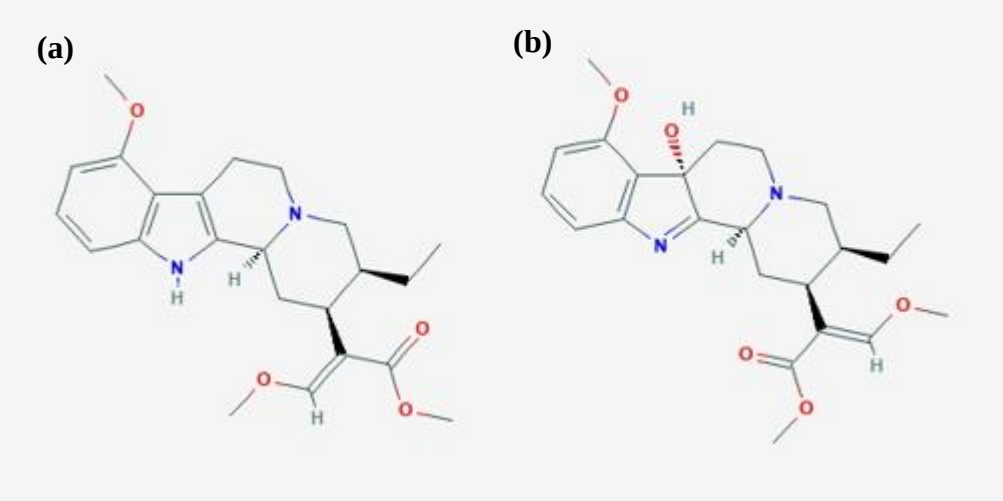

Figure 2. 1: Chemical structure (a) mitragynine and (b) 7-hydroxymitragynine compound retrieved from National Center For Biotechnology Information (2020)

Previously, Houghton & and Said (1986) reported new alkaloid isolated from the Malaysian mitragyna speciosa plant. Four new alkaloids were found in younger ketum leaves which were mitragynine, corynantheidaline, mitragynalinic acid, and corynantheidalinic acid. Related to the study, Takayama (2004) isolated 5 alkaloids which were mitragynine, speciogynine, speciociliatine, paynantheine, and 7-hydroxymitragynine from methanolic extraction mature leaves from Thailand plants.

Mitragynine compound is the main and major alkaloid that are presence in ketum leaves and gives a major effect on the user's body. Despite that, another minor alkaloid compound i.e. 7-hydroxymitragynine would also affect the psychology and physiology of the ketum takers and has been identified to be more potent than the mitragynine compound (Matsumoto, et al., 2004).

2.3 Trends and Law Enactment

Ketum is the coffee family tree that growth abundantly in Southeast Asia such as Malaysia, Thailand, and Burma (Myanmar). For the past few decades, ketum was used for treated common diseases such as diabetes and high blood pressure that act as herbal medicine and herbal remedy. The use of ketum in old folks and hard labour worker was famous to retreat, suppress fatigues and combat strength due to hard labour (Suwanlert, 1975; Watanabe, et al., 1997; Vicknasingam, et al., 2010; Hassan, et al., 2013). However, the trends are now shifting from herbal remedy to abuse among youngsters. They took ketum for their pleasure by addition of other substances that may enhance the opioid properties in ketum products and made as an alternative or substitution of opioid type of drugs (Singh, et al., 2016).

Ketum plants consist of psychoactive substances which are grouped in new psychoactive substance (NPS) in the category of plant-based psychoactive substance due to mitragynine and 7-hydroxy mitragynine that had properties similar to other opioid drugs. Ketum was prohibited in certain countries such as Malaysia, Thailand, and Australia. In Malaysia ketum was regulated under the Poison Act 1952 stated in Schedule 3 section 30 which stated that mitragynine as psychotropic substances. Under this schedule, any person who is importing, exporting, manufacturing, and selling of ketum be liable to a fine not exceeding ten thousand ringgit or to imprisonment for a term not exceeding four years or both. However, planting of ketum tree was not an offense and the tree grows widely in this country which hard to control. The use of ketum among communities in Malaysia is widely spread and available to access.

Furthermore, ketum also was banned in countries such as Thailand, Myanmar, and Australia. In Thailand around 1943, ketum/ kratom was the first schedule for control under the Kratom Act and the law enforcement was lenient at that time. Later, in 1979 ketum was rescheduled and include in the Thai Narcotics Act under Schedule 5 which least restrictive and punitive level (Tanguay, 2011). Traditionally, ketum was used as part of the culture in the Thailand community. They were taking ketum by chewed and considered chewing ketum like drinking a coffee. But, the used trend of ketum had emerged by boiling the leaves as tea with the addition of cough syrup, Coca-cola, and ice cubes that produced a basic 4x100 cocktail (Tanguay, 2011).

Ketum was also prohibited and banned in countries such as Australia and Myanmar. Australia is regulated ketum and mitragynine under Schedule 9 of the Australian National Drugs and Poison Schedule and in Myanmar under section 30 of the Narcotic Drug and Psychotropic Substance Law 1993. Other countries such as the USA and the UK, the ketum legally used and can easily access online.

The legal status of ketum in European countries varies from region to region. In Denmark, Finland, Ireland, Latvia, Lithuania, Poland, Romania, and Sweden, the status of ketum is illegal (Guide, 2018). While, in the United Kingdom, the legal status of ketum is debatable. Before UK Psychoactive Substance Act come into effect, ketum was illegal to import, export, or sell in the UK. In May 2016, ketum is included in the list of outlawed substances that produce psychoactive effects under the UK Psychoactive Substance Act (Guide, 2018).

2.4 Extraction methods

Extraction is the prior step for the identification of the target compound from the bulk or actual samples in forensic investigation or examination. The extraction

mitragynine from Ketum leaves needs to perform using several methods that are suitable for all sample matrices (biological and non-biological). Extraction that needs to perform can be simple as dilute the sample with methanol which is called simple extraction or need to be modified and undergoes simple or a long and tedious extraction process.

2.4.1 Simple Extraction

According to Casey, et al. (2015), for extract the ketum sample the researchers used simple extraction by diluting the liquid sample with methanol and then filter using a 0.2 µm PTFE filter. The total volume of the mixture before the filter is about 10 ml. The aliquot was kept and ready to analyse by GC/MS. These extraction methods only provide simple procedures and involve only methanol as a solvent for extracting the mitragynine in liquid samples. However, the colour test for the sample was not mentioned in the study.

2.4.2 Acid and Base Extraction

The extraction method for non-biological samples specifically liquid samples e.g. ketum drinks were using the acid-base extraction method by Chan, et al., (2005). This method was established extraction method for liquid samples of seized ketum drink samples. According to this study, the methods required liquid samples acidified with concentrated HCL and basified with NaOH solution after discarded the ether layer. Then, chloroform was added in the solution twice and filtered through anhydrous sodium sulphate.

The extraction method provides by Chan, et al. (2005), is the established extraction method used in forensic investigation in Chemistry Department, Malaysia to analyse the liquid samples of seized ketum product intended for instrumental

analysis. These methods require acidic and basic chemicals for extracting out the mitragynine compound in the liquid samples for example from ketum tea or 'cokoroi' (ketum concoction drinks). The extracted sample will be reconstituted in methanol solution and ready for analysis in the gas chromatogram (GC) for confirmatory analysis. Again, this extracted solution was not subjected to any colour test before instrumental analysis.

2.4.3 Extraction Procedure for solid samples

Ketum samples submitted to forensic laboratory were categorized in different forms which are in the form of solid such as powdered or raw material, in liquid or concoction samples, and also biological samples such as urine or blood. The extraction method for solid samples such as powdered and raw material was performed by Kikura-Hanajiri, et al. (2009). In their researches, they study the quantitative analysis of commercial ketum samples in the form of solid samples (powdered) and raw material.

This extraction of mitragynine in solid raw material and commercial ketum samples may come in handy because the most established methods for extracting the mitragynine compound was in the form of liquid samples. Kikura-Hanajiri, et al. (2009) performed an extraction procedure by dissolved the solid sample (powder) in 80% methanol and internal standard then ultrasonicated for 1 hour. Then, the ultrasonicated sample was kept in overnight and filter through a filter device. The extracted mitragynine product was diluted with methanol in a suitable concentration for further analysis.

Other studies also involved in the extraction mitragynine from the solid sample was made by Chan, et al. (2005). According to Chan, et al. (2005), the powder ketum

product was ultrasonicated in the mixture of methanol and chloroform about a ratio of 1:4 for 10 minutes. Then aliquot was taken for further analysis for confirmatory analysis or qualitative analysis.

2.4.4 Phytochemistry extraction

The phytochemistry procedure is a procedure involving plant materials that followed a few steps which are extraction of plant material, identification of phytoconstituents, separation and isolation of phytoconstituents, and also the characterization of isolated compound. The compounds found in plants are of many kinds, but most are in four major biochemical classes i.e. the alkaloids, glycosides, polyphenols, and terpenes.

Ketum plants contain the biochemical compound, alkaloids which are mitragynine and other compounds. Thus, the extraction of mitragynine using phytochemistry techniques is far more suitable to identify the target compound in ketum plants. There was established phytochemistry methods for extracting mitragynine from original state (plants and leaves) was made by Kikura-Hanajiri, et al. (2009).

According to Kikura-Hanajiri, et al. (2009), the powdered sample was extracted with 80% methanol aqueous solution and adding an internal standard solution, then ultrasonication for 1 hour. After stored overnight, the sample was centrifuged at 3000 rpm for 5 min. Then, filtered through a centrifuge filter device.

2.4.5 Soxhlet extraction

Isolation and extraction of pure mitragynine are crucial processes identifying mitragynine compounds in ketum products. There are several methods for extraction of mitragynine in ketum plants and one of them is Soxhlet extraction. Soxhlet

extraction may produce pure mitragynine from ketum leaves but it was time-consuming and the process was lengthy and laborious for extracting pure compounds from ketum leaves.

According to Beng, et al. (2011), the isolation of mitragynine using Soxhlet extraction may require a slow process and takes days to obtain a crude extract of ketum plants. The extracted product (crude) obtained contains pure mitragynine compound, however, the crude extract obtained was sticky, agglutinated, and might need to sonication to facilitate the extraction of mitragynine (Beng, et al., 2011).

2.4.6 Extraction methods for biological samples

Forensic drug investigation does not only cover the seized sample or bulk samples taken from the crime scene. It also involves forensic toxicological drug screening to identify and detect the presence of drugs or substances in the user's body, which means the by-product and its metabolites. Generally, identifying or detecting the presence of the target compound, the investigator taking biological samples such as urine, blood, or saliva at the crime scene and also suspect.

Biological samples come in handy when performing the extraction for extracting out the target compound. Most of the time, the target compound does not present in its true chemical compound or composition. The compound will metabolise in the body and result in metabolites compound. This means the chemical compound or composition changed slightly or change thoroughly making to a new compound. Sometimes, the metabolites difficult to extract out from the sample due to complex structure and composition.

The extraction of biological samples undergoes several steps and processes by adding certain chemicals or enzymes for extracting the target compound from

biological samples. Identification of active compounds; mitragynine in biological samples especially urine may undergo a few steps and phases. Several studies had performed analyses of mitragynine detection in the urine samples. They performed hydrolysis using þ-glucuronidase and arylsulfatase due to metabolites that were mostly excreted in conjugated form. Regarding the extraction procedure, some authors perform liquid-liquid extraction with methyl tert-butyl ether (Philipp, et al., 2011; Lu, et al., 2009), ethyl tert-butyl ether (Guddat, et al., 2016) or n-butyl chloride (Holler, et al., 2011).

The extraction of MG from biological sample i.e. urine was carried out following in-house methods developed by Pharmacology Department in HUSM Health Campus. Urine sample is adjusted with 0.2M sodium hydroxide to obtain pH 8.5±0.1. Then 10mL MTBE is added into the urine sample and shaken with orbital shaker at 250rpm for 15 mins. The MTBE is collected and dried using purified nitrogen air at 45°C to dryness. Methanol is used to reconstitute the analyte.

Although there are reported and proved that the method mentions above were reliable and produce a sufficient amount of mitragynine in urine, the methods are tedious, too laborious, and not cost-effective due to use of highly specialised enzymes. Further studies need to be carried out for in-expensive and rapid detection for screening mitragynine in a urine sample and other biological samples (blood and saliva).

2.5 Preliminary test using colour spot test

As for forensic drug investigation, there is a standard drug protocol that requires two steps analysis and one of them is preliminary testing. One of the preliminary testing is a colour test for the identification of any favourable substance

or drugs. The colour test is screening and first process for detection of any drugs in forensic investigation. The colour that changes using a specific reagent kit or reagent has been observed and recorded.

Preliminary testing can result in two possible outcomes which are false positive and false negatives. In a forensic investigation, outcomes and results of the investigation give impact to suspect or individual that has been charged. Preliminary testing cannot charge the person guilty, however, it is the first testing before a forensic drug investigation.

Ketum samples that have been submitted to the forensic lab may come in different types and preliminary testing for detection of mitragynine would be tricky. Mitragynine can be detected using the Van Urk colour test. Even though there is not reported the success rate of Van Urk spot test for the detection of mitragynine in real sample cases, it probably can be used due to the presence of indole compounds in ketum plants (Chan, et al., 2007).

Van Urk colour test or Van Urk reagent test is a sensitive and specific chromogenic reagent for the detection and identification of indole derivatives. Van Urk reagent would produce a colour reaction that had a wide spectral range from yellow to blue and extremely stable (Ehmann, 1977). The sensitivity and specificity of Van Urk reagent would comply for detection of mitragynine.

On the other hand, some studies reported the testing of other colour tests for detection of mitragynine from seized ketum samples and also commercialize ketum products that had been legally sold online. Scott, et al. (2014) reported on their studies about the reactivity of Duquenois-Levine test that is commonly used for presumptive identification of marijuana towards commercial ketum product which sold online and

other colour spot test: nitric acid, Liebermann, Marquis, Froedhe, Mecke, and Simon's tests. The result shows a non-specific colour reaction for all the colour tests that tested thus, the group of tests not significantly useful in presumptive identification of mitragynine.

Similarly, Chan, et al. (2007) performed a screening test of dry and powdered ketum leaves using the Duquenois-Levine test for detection of mitragynine. The result showed a dull blackish green colour which not extractable into the chloroform layer. Chan, et al. (2007) reported that other colour tests would possibly react with mitragynine's indole moiety, for example, Van Urk, Ehrlich, and Wasicky reagent. However, those tests were not performing in their present work.

Previously, a colour test using Van Urk reagent for detection of mitragynine was performed by Mat Ghani (2017) and Mohamad Zamri (2019) shown the positive result which was the pink colour reaction. The small amount of van Urk reagent was dropped in the sample and produced a pink colour reaction. Testing is challenging especially when submitted samples were already in dark colour; thus, it is difficult to see any reaction with the reagents.

2.5.1 Spectrophotometry colorimetric analysis

The colorimetric analysis is a technique that is usually used to determine the concentration of the analyte by comparing the colour changes of the solution. Determination of concentration of colour changes using colorimetric analysis can be performed for the identification of drugs. In this case, identification mitragynine from ketum product and ketum raw material also can be done using spectrophotometry colorimetric analysis since, identification of mitragynine results on colour changes when reacting with certain reagents such as Van Urk, Ehrlich, or Wasicky reagent.

However, there is no study of colorimetric analysis using a smartphone as a detector for the identification of mitragynine compound.

Colorimetric analysis using a smartphone camera as a detector is a fast and easy technique which allowed our smartphone as a device to generate data and analyse sample. A few studies reported the use of a smartphone camera as an instrument of spectrophotometric analysis for quantification of analyte concentration and produce rapid results (Scheeline, 2010). This journal describes how cell phones and smartphone can be used to perform colorimetrically (Koesdjojo, et al., 2015; Kehoe & and Penn, 2013) and fluorescence analyses (Koenig, et al., 2015).

The purpose of doing colorimetric analysis using smartphones as a detector for detections of mitragynine is can produce rapid, cost-effective, and accessible for field testing. According to a few studies, mobile phones/ smartphones can produce the best result in the quantification of analyte concentration. There are a few smartphone app algorithms that were created that compatible with both iOS and Android users to quantify colorimetric testing. The main purpose of creating mobile apps that provides rapid on-site quantitative screening for fast results is needed (Yetisen, et al., 2014).

The standardized and conditioned setting needs to be done to produce an accurate and ideal result for colorimetric testing using smartphone apps. According to Solmaz, et al. (2018), the quantification of the colorimetric test was done based on machine learning classifies. The experimental design and image capture were designed that involves controlled condition including illumination and distance between the detector (smartphones) with samples. The smartphone app was used to measure the RGB values from the image taken by the camera phones.

2.6 Munsell colour System

Colour theory or colour system was made to describe the colour more specifically and analyse the colour according to numerically value. The colour system was been used in industrial, fashion, and including forensic investigation. In forensic investigations, colour test or colorimetric analysis was one of the analyses that involve in different colours and related to the concentration.

Colour was described and interpreted differently between individuals even though it was the same colour. To interpret the specific colour, the Munsell colour theory was introduced which consists of two-part which were colour chart and theoretical system. Munsell colour chart helped to describe the colour based on three variables: hue (H), value (V), and chroma (C). Hue defines as actual 'colour' that follow the natural basic colour of red (R), yellow (Y), green (G), blue (B), and purple (P). While, in between the colour were intermediated colour such as yellow-red (YR), green-yellow (GY), blue-green (BG), purple-blue (PB), and purple-red (PR). Value is the lightness of colour (range from 0 for pure black to 10 for pure white) and chroma is the degree of departure of colour from the neutral colour of the same value. Munsell organized the colour sample into 3-dimensional spectra solid shape with hue, chroma, and value as the axes (Cochrane, 2014).

2.7 Presumptive test using Thin Layer Chromatography (TLC)

The alkaloid composition of botanical and forensic samples can be analysed by regular chromatographic and spectroscopic methods (EMCDDA, 2020). The thin-layer chromatography technique can separate alkaloid compounds from ketum plants on silica gel plates with detections of low UV light (256 nm). Mitragynine gives colour purple or grey-to-brown spots when spraying with modified Ehrlich's reagent or ferric

chloride-perchloric acid reagent unto the developed TLC plate. Identifications of mitragynine in plant material (ketum plant) have been achieved using standard TLC methods and high-performance thin-layer chromatography (HPTLC).

Identification of mitragynine and another constituent in ketum products and other legal high products that had been sold online was been studied by Scott, et al. (2014) using TLC techniques. According to Scott, et al. (2014), the TLC silica plates that were spotted with mitragynine standards and samples were developed with a solvent system of 9:1 chloroform/methanol. Then, the plate was visualized under UV light at 254 nm wavelength followed visualization spray of iodoplatinate. Upon visualized with iodoplatinate, the bands of mitragynine and samples turned purple colour and 30 min later change to yellow, than when allowed to sit overnight the colour change to orange. Mitragynine obtains an average Rf value of 0.79 results on TLC techniques. (Scott, et al., 2014).

Kowalczuk, et al. (2013) proposed the comprehensive authentication method for botanical analysis of plants material and rapid yet inexpensive identification of mitragynine using thin-layer chromatography (TLC) and HPLC. TLC silica plates were developed in a solvent system of hexane/ethyl acetate/25% ammonia solution (30:15:1 v/v/v) then examined under UV light (254 nm). Results revealed the presence of mitragynine which presence of pink-grey spot with Rf value 0.49 when observed under UV-Vis lamp in the wavelength of 254 nm.

Beng, et al. (2011) proposed the study to develop a simple and inexpensive method for extraction of mitragynine from ketum leaves. The qualitative identification of isolated mitragynine was carried out via analytical methods such as high-performance thin-layer chromatography (HPTLC). HPTLC was performed on a silica

gel plate using hexane: ethyl acetate (80:20 v/v) as a mobile phase. Then the plate was visualized under UV light of 254 and 366 nm wavelength. Results from the TLC study shown the value of hRf is 81.9. This value was relatively mean the value of isolated and purified mitragynine.

2.8 Instrumental confirmatory tests

Standard drug protocols for any forensic drug examination undergo preliminary and also confirmatory analysis to identify the presence of drugs. Ketum plants were categorized under new psychoactive substances which is the plant that can give psychoactive effects to the users. In real cases, ketum samples that were seized then submitted to the forensic laboratory were in the form of bulk ketum leaves and also liquid concoction. Identification and quantification of mitragynine which is the main alkaloid compound in the Ketum leaves are important to identify whether the compound presence or absent in the submitted sample.

To date, quantification and identification of isolated and extracted mitragynine from ketum leaves had been achieved with the following methods: TLC and high-performance liquid chromatography (HPLC) (Kowalczuk, et al., 2013), gas chromatography techniques with flame ionization detector (GC-FID) (Chan, et al., 2007), more advance techniques such as ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (Sharma, et al., 2019). On the other hand, identification and quantification of mitragynine in biological samples (urine) can be achieved by liquid chromatography-tandem mass spectrometry was performed by (Fu, et al., 2015).

Chromatography techniques were the most accepted techniques for identifications of mitragynine alkaloids and other constituents in ketum leaves and

ketum product (Wang, et al., 2014). Wang, et al. (2014) studied the comparison of three different techniques of detection of mitragynine compound in ketum leaves. The researchers found that the most famous chromatographic technique for identifications of mitragynine and had proven the effectiveness was by the HPLC technique.

2.8.1 Gas Chromatography

The gas chromatography method had been used for identifications and quantifications of mitragynine and other alkaloids in ketum leaves and its product for many years. The gas chromatography method was one of the common methods that been published for detecting mitragynine from ketum samples and ketum leaves (Wang, et al., 2014). However, there were some pros and cons regarding this technique that were used for quantification and detection of mitragynine.

Chan, et al. (2005) identify the mitragynine in ketum leaves and ketum preparation using gas chromatography techniques with mass spectrometry and flame ionization detector. This was the earliest GC method for analysis of mitragynine for forensic drug investigation along with the problems of ketum used among Malaysians. In this study, the mitragynine from various samples ie liquid and solid samples were identified using GC-FID and GC/MS by comparing samples with standard reference. The column used for GC-FID was utilized HP-5 capillary column with a thickness of 0.25 µm and the temperature-programmed was relatively high about 200° C to 300 ° C. Results showed the peak for mitragynine was <17 min relatively by comparison of standard mitragynine in the library spectra.

Philipp, et al., 2011 used gas chromatography techniques for monitoring ketum or Krypton intake in the urine. The detection of mitragynine in urine identifies the presence of ketum intake after the metabolic and enzymatic process. By comparing

the published methods using LC-MS (Philipp, et al., 2009) for detecting mitragynine and its metabolites in human urine, mitragynine and its metabolite can only be detected using GC/MS.

2.8.2 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography is the chromatography technique that successfully and popular methods for the analysis of ketum alkaloids. The identification of mitragynine may require chemical and instrumentation that difficult for interpretation. However, Kowalczuk, et al. (2013) proposed the comprehensive method that rapid and economical methods using HPLC for detections of mitragynine compounds in various ketum product. HPLC method provided a much shorter retention time for mitragynine (11 minutes) and shorter total analysis time (15 minutes). In this study, they demonstrated methods for good separation, high sensitivity, and low limit of detection values of mitragynine. This method had been applied for identifications of ketum samples and products in police laboratories.

Quantification of mitragynine in ketum raw material and end products was done by Mudge & and Brown (2017) using high-performance liquid chromatography with UV detection. This method was suitable for the detection and identification of mitragynine, 7-OH mitragynine, and another alkaloid in ketum raw material and finished product. HPLC-UV method is a reliable method for quantification of alkaloid in ketum plants however, there have limited resolution and different compositions of the mobile phase, stationary phase, and run times.

Kikura-Hanajiri, et al. (2009) studied the simultaneous analysis of mitragynine, 7-hydroxyl-mitragynine, and other indole alkaloids in ketum leaves using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-

MS). This method provides reliable results regarding detections of target compounds and showed absents of interference with almost any targeted compounds. Besides, the researchers measured the actual content of alkaloids in ketum products. However, this method provided a much higher cost for instrumentations and not all laboratories equipped for such high-end instrumentation.

Mitragynine and other alkaloids that present in ketum leaves or other ketum products can be detected using a few techniques and methods. However, identification of mitragynine in urine specifically human urine may introduce other alkaloids or metabolites that difficult for data interpretation and identification of target compounds. Philipp, et al. (2009) using liquid chromatography ion trap mass spectrometry for the metabolism of mitragynine in rat and human urine. This technique enables us to provide information about the metabolism of alkaloids in the human body from the urine taken for analysis. However, the method involves two-phase of metabolism which may require a series of extractions procedure for extracting the mitragynine from the urine and the instrumentation was higher in cost compare to standard chromatography techniques.

Janchawee, et al. (2007), studied about HPLC method for determining mitragynine in rat serum and applied to a pharmacokinetic study. In this method, the mitragynine extracted from ketum leaves by simple liquid-liquid extraction resulting in a high recovery of the analyte. According to Janchawee, et al. (2007), this method provides simples, sensitive, precise, and accurate result for serum analysis. This study investigates mitragynine from various samples toward its colour reaction with Van Urk reagent.

CHAPTER 3

METHODOLOGY

3.1 Sample collection, reagent and materials.

Fresh white vein leaves of *Mitragyna speciosa* (ketum) were collected from Kelantan. Anhydrous sodium sulphate, chloroform, concentrated hydrochloric acid (HCL), ethanol, methanol from Merck (Germany) and para-Dimethylaminobenzaldyde from Merck Germany; and sodium hydroxide (NaOH) was from Friedemann Schmidt (Australia). Distilled water from laboratory supply was used throughout the procedure. Pholcodine cough syrup (Tussedyl Forte Syrup, Malaysia) was brought at nearest local pharmacy and the Coca-Cola drinks (Malaysia) was bought at local supermarket.

3.2 Apparatus

Apparatus used in this study include beaker, Scott bottles and glass evaporating dish from Schott Duran (Germany); measuring cylinder and volumetric flask from Favorit (Germany); and separating funnel from Glassco (UK). Polypropylene PCR Microplate containing 96 wells was obtained from HID Unit, Universiti Sains Malaysia, Kubang Kerian Kelantan. Clean and sanitized urine containers was supplied by Hospital Universiti Sains Malaysia, Kubang Kerian, Kelantan. An advanced activated carbon filtration bottle was obtained from OKO (US).

3.3 Instruments

The spectrometry analyses were carried out using SpectraMax M5^e Series Multi-Mode Microplate Reader at Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, Kubang Kerian, Kelantan. The analysis was conducted at INFORMM and the instrument was provided by INFORMM under